



Quinolizidine alkaloids are transported to seeds of bitter narrow-leaved lupin

Otterbach, Sophie Lisa; Yang, Ting; Kato, Lucilia; Janfelt, Christian; Geu-Flores, Fernando

Published in:
Journal of Experimental Botany

DOI:
[10.1093/jxb/erz334](https://doi.org/10.1093/jxb/erz334)

Publication date:
2019

Document license:
[CC BY-NC](#)

Citation for published version (APA):
Otterbach, S. L., Yang, T., Kato, L., Janfelt, C., & Geu-Flores, F. (2019). Quinolizidine alkaloids are transported to seeds of bitter narrow-leaved lupin. *Journal of Experimental Botany*, 70(20), 5799-5808.
<https://doi.org/10.1093/jxb/erz334>

RESEARCH PAPER

Quinolizidine alkaloids are transported to seeds of bitter narrow-leaved lupin

Sophie Lisa Otterbach^{1,2}, Ting Yang^{1,2}, Lucilia Kato^{3,†}, Christian Janfelt³ and Fernando Geu-Flores^{1,2,*}

¹ Section for Plant Biochemistry, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Denmark

² Copenhagen Plant Science Centre, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark

³ Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

[†] Present address: Instituto de Química, Universidade Federal de Goiás, Campus Samambaia, Goiânia, 74690–900, Goiás, Brazil

* Correspondence: feg@plen.ku.dk

Received 6 May 2019; Editorial decision 8 July 2019; Accepted 11 July 2019

Editor: Robert Hancock, The James Hutton Institute, UK

Abstract

Narrow-leaved lupin (NLL, *Lupinus angustifolius*) is a promising legume crop that produces seeds with very high protein content. However, NLL accumulates toxic quinolizidine alkaloids (QAs) in most of its tissues, including the seeds. To determine the level of *in situ* biosynthesis in the seeds, we compared the accumulation of QAs with the expression of the biosynthetic gene lysine decarboxylase (LDC) in developing seeds and pods of a bitter (high-QA) variety of NLL. While QAs accumulated steadily in seeds until the drying phase, LDC expression was comparatively very low throughout seed development. In contrast, both QA accumulation and LDC expression peaked early in pods and decreased subsequently, reaching background levels at the onset of drying. We complemented these studies with MS imaging, which revealed the distribution patterns of individual QAs in cross-sections of pods and seeds. Finally, we show that a paternal bitter genotype does not influence the QA levels of F₁ seeds grown on a maternal, low-QA genotype. We conclude that the accumulation of QAs in seeds of bitter NLL is mostly, if not exclusively, transported from other tissues. These results open the possibility of using transport engineering to generate herbivore-resistant bitter NLL varieties that produce QA-free seeds.

Keywords: *iucundus* locus, long-distance transport, *Lupinus angustifolius*, MALDI-MSI, maternal determination, metabolite imaging, narrow-leaved lupin, quinolizidine alkaloids.

Introduction

Lupins (*Lupinus* spp.) are genistoid legumes with an untapped agronomic potential. As nitrogen fixers, lupins can enrich the soil and reduce the incidence of disease in subsequent cereal crops (Sweetingham and Kingwell, 2008). In addition, lupins produce grain with a unique composition: high in protein (~40%), low in oil (~10%), and virtually no starch (Petterson, 2016). This favorable composition makes lupin grain attractive for both food and feed applications. In Europe, lupins are

among the candidate protein crops that may help curb the European Union's notorious dependency on imported soybean for use as animal feed (de Visser *et al.*, 2014). With respect to food applications, lupins represent an excellent option for replacement of both animal and gluten-containing products (Lucas *et al.*, 2015). Interestingly, consumption of lupin grain has been associated with significantly reduced blood glucose levels after meal intake, particularly in individuals with type II

Abbreviations: DAA, days after anthesis; LDC, *lysine decarboxylase*; MALDI-MSI, matrix-assisted laser desorption/ionisation-mass spectrometry imaging; NLL, narrow-leaved lupin; QA, quinolizidine alkaloid; qRT-PCR, quantitative reverse transcription PCR; TUB, β -Tubulin; UBC, Ubiquitin C.

© The Author(s) 2019. Published by Oxford University Press on behalf of the Society for Experimental Biology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

diabetes (Dove *et al.*, 2011; Fornasini *et al.*, 2012; Bouchoucha *et al.*, 2016; Schopen *et al.*, 2017).

Despite these positive attributes, lupins are far from living up to their full potential. In Europe, the yield of lupin is generally lower than that of several other soybean alternatives, in particular faba beans and peas (de Visser *et al.*, 2014). This can be partly attributed to a relatively recent domestication history, which has yet to exploit the rich genetic material available within many unexplored wild varieties and landraces (Berger *et al.*, 2012; Atnaf *et al.*, 2017). Future genetic enhancements are expected to yield varieties with improved traits, such as enhanced disease resistance, adaptation to calcareous soils, and resistance to summer drought and soil salinisation (Lucas *et al.*, 2015). One key trait that will require particular attention is the accumulation of anti-nutritional compounds in the grain. Wild varieties and landraces can accumulate up to 5% of their dry weight in the form of alkaloids, most of them belonging to the family of quinolizidine alkaloids (QAs) (Frick *et al.*, 2017). QAs are notoriously bitter and toxic to both humans and farm animals, displaying both teratogenic and anti-cholinergic effects (Lourenço *et al.*, 2002). Accordingly, traditional consumption of lupin grain involves a debittering process where the grain is boiled and subsequently subjected to extensive leaching in water or brine to remove QAs. The process is cumbersome and also removes a large proportion of soluble proteins, minerals, flavonoids, monosaccharides, and sucrose from the seeds (Erbas, 2010).

In order to bypass the debittering process, breeders have developed cultivars with low-QA content. The development of these 'sweet' cultivars can be traced back to the late 1920s, when the German breeder Reinhold von Sengbusch identified the first low-QA individuals of narrow-leaved lupin (NLL, *L. angustifolius*), white lupin (*L. albus*), and yellow lupin (*L. luteus*) by screening very large plant populations (von Sengbusch, 1942). In the late 1960s, Australian breeders developed the first commercially successful 'sweet' cultivars by combining the low-QA trait (sweetness) with fundamental agronomic traits, such as early flowering and resistance to pod shattering (Cowling and Gladstones, 2000). Thanks to the breeding programme led by John S. Gladstones, Australia became the main producer of lupin grain worldwide in the 1980s, a position that they have maintained since (FAOSTAT, 2018).

Despite the obvious advantages of sweet cultivars, lupin cultivation has not picked up significantly in Europe. At the same time, the total cultivated area used for lupins in Australia has been in decline since the late 1990s (FAOSTAT, 2018). One of the reasons for this is that grain from sweet cultivars is not free of QAs and, depending on the harvest year and location, the QA levels might exceed industry thresholds (0.02% DW for animal feed and 0.01% DW for human consumption) (Cowling and Tarr, 2004; Frick *et al.*, 2018). The relationship between environmental factors and grain QA levels is not only complex, but also cultivar-specific, adding a layer of complexity to the problem (Cowling and Tarr, 2004; Frick *et al.*, 2018). At the same time, it is known that sweet cultivars

are more susceptible to herbivores than bitter varieties (Wink, 1991; Kozłowski *et al.*, 2017). This is not surprising considering that QAs can exert a variety of protective effects against insects, aphids, and snails (Dreyer *et al.*, 1985; Wink, 1991; Berlandier Françoise, 1996), and that sweet varieties are not only low in QAs in the grain, but also in vegetative tissues. Aware of the inverse relationship between vegetative QA levels and aphid susceptibility, the Australian breeding programme originally used a strategy to select for best possible resistance to aphids while simultaneously selecting for least possible QA content in the grain. However, their efforts failed to produce sweet cultivars with sufficient bitter foliage to deter aphids (Lee *et al.*, 2007). Despite this disappointment, the idea of obtaining a bitter-sweet phenotype (bitter in vegetative tissues and sweet in the grain) lived on in the writings of Professor Michael Wink, who pioneered biochemical research on QAs in the 1980s and who was the first to propose the use of biotechnology to achieve this goal (Wink, 1991).

In order to develop strategies for the generation of bitter-sweet cultivars, a much better understanding of QA biosynthesis and translocation is needed. One outstanding question concerns the extent to which QAs are synthesised *in situ* in the developing seeds, in contrast to being transported from other tissues. The biosynthesis of QAs has not yet been fully elucidated, but the first step is known to be catalysed by the enzyme lysine decarboxylase (LDC) (Bunsupa *et al.*, 2012). In bitter cultivars/accessions of NLL, LDC is expressed at high levels in leaves, stems, pedicels, and pods, and at low levels in roots, flowers, and seeds (Yang *et al.*, 2017; Frick *et al.*, 2018). At the same time, QAs are highly abundant in the phloem of bitter lupins (up to 20 mM) (Wink *et al.*, 1982; Wink and Hartmann, 1982; Lee *et al.*, 2007), which is consistent with the possibility of long-distance transport. Indeed, when deuterated versions of QAs are fed to white lupin using the cut-stem method, the labelled compounds are recovered in pods, leaves, stems, petioles, and seeds (Lee *et al.*, 2007). In order to establish the extent to which QAs are transported into white lupin fruits, Lee *et al.* (2007) performed theoretical calculations based on sap composition and water economy, and arrived at the conclusion that half of the total amount of QAs in fruit tissues was derived from transport processes and half was synthesised *in situ*.

In the present study, we set out to investigate the extent of *in situ* biosynthesis in developing seeds of bitter NLL. We followed the expression of LDC and the accumulation of QAs in seeds and pods of a bitter cultivar using quantitative reverse-transcription PCR (qRT-PCR) and LC-MS, respectively. We complemented these studies by visualising QAs in tissue sections of seed-containing pods at two selected time-points using matrix-assisted laser desorption/ionisation imaging (MALDI-MSI). In addition, we conducted a crossing experiment to assess the impact of a bitter, paternal genotype on the QA levels of F₁ seeds grown on a maternal, sweet genotype. Our combined results strongly suggest that most, if not all, of the seed QA levels in bitter NLL are translocated to the seed via transport processes.

Materials and methods

Plant material and growth conditions

Seeds of narrow-leaved lupin (NLL, *Lupinus angustifolius*) cv. Oskar (bitter) were obtained from Hodowla Roślin Smolice Sp. z o. o. Grupa IHAR (Smolice, Poland). Seeds of NLL cv. Iris (sweet) were obtained from Plantefrø.dk (Frederiksberg, Denmark).

Seeds were hand-sown in a 100-m² field at the Department of Plant and Environmental Sciences, Taastrup Campus, Copenhagen, Denmark, in 2016, leaving 30 cm between plants in each row and 50 cm between rows. Each seed was planted together with ~15 g of peat that had been pre-inoculated with a resuspension of *Bradyrhizobium lupini* strain from the commercial product RADICIN-Lupin (JOST GmbH, Iserlohn, Germany). The field-grown plants did not receive additional watering and were not sprayed against pests. Weeding was carried out manually.

Sampling of tissues during seed development

For each individual, the approximate day of anthesis was recorded (Dracup and Kirby, 1996), and sampling commenced 10 d after anthesis (DAA). For each of the seven time-points, the three first pods from the primary stem were harvested together with a young leaf close to the inflorescence. The sampling was carried out in the morning and included six biological replicates, which were later used for alkaloid analysis, gene expression analysis, and three biological replicates for determination of water content. Plant material was weighed, frozen in liquid nitrogen and stored at -80 °C until further analysis. Pods were weighed separately from seeds, and seed weight was recorded after pooling all seeds from one pod. Water content was determined after freeze-drying.

Crossing of NLL bitter and sweet varieties

Plants were crossed as follows: NLL cv. Iris (maternal plant) × NLL cv. Oskar (paternal plant), and NLL cv. Iris (maternal plant) × NLL cv. Iris (paternal plant) as control. Either the first, second, or third flowers to emerge from the maternal plants were used in the crosses. Flowers were emasculated at their immature stage (when the flower buds were still hooded by the calyx) without removal of any petals, and 2 d later they were fertilised with ripe pollen. Entire pods were harvested upon reaching full maturity (~85 DAA) and stored in dry and dark conditions. The crosses were conducted in the field in two independent experiments at the Department of Plant and Environmental Sciences: the first in 2016 on the Taastrup Campus, and the second in 2017 on the Frederiksberg Campus (both Copenhagen). Pods resulting from successful crossing events were identified by sowing one seed per pod and identifying bitter seedlings (high in QAs) (Supplementary Fig. S3 at JXB online). The remaining seeds in the positively identified pods were then analysed for their QA content. For the control cross, we also sowed one seed per pod, and no resulting seedlings were found to be bitter (Supplementary Fig. S3 at JXB online).

Gene expression analysis

RNA extraction and cDNA synthesis.

Harvested tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was isolated from ~100 mg of tissue using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich), including on-column DNase digestion. After observing the two main ribosomal RNA bands on an agarose gel, the quality and quantity of RNA was assessed using a Nanodrop™ spectrophotometer ($A_{260}/A_{230} > 1.8$ and $A_{260}/A_{280} > 2.0$). cDNA was obtained from 1 µg of RNA in 20-µl reactions using the SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific) with oligo (dT)₂₀ and random hexamer primers in a 1:1 ratio. All cDNA samples were diluted 10-fold before transcript quantification by qRT-PCR.

Primer design and testing.

Primer pairs were designed for *Lysine decarboxylase* (LDC) and β -*Tubulin* (TUB) using sequences identified from the transcriptome of *L. angustifolius* cv. Oskar (Yang et al., 2017). For *Ubiquitin C* (UBC), we used sequences designed and tested by Taylor et al. (2016). All primer sequences are presented in Supplementary Table S1. Evaluation of primer efficiency, dynamic range, and specificity was carried out using a serial dilution of a pool of cDNA samples from leaves, pods, and seeds. For this evaluation, the qRT-PCR protocol described below was used. Control reactions with template RNA that did not undergo reverse-transcription (minus RT controls) gave no product for the TUB and UBC primers. Similar controls using LDC primers gave a product of very low abundance at C_T values between 37–38, which was 5–6 units higher than the highest sample C_T value of 32. All primer efficiencies ranged between 95–98%.

Transcript quantification by qRT-PCR.

Quantitative reverse-transcription PCR (qRT-PCR) was performed and analysed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Inc.) and accompanying Bio-Rad CFX Manager software (ver. 3.1). Each qRT-PCR reaction contained 2 µl cDNA (1:10 dilution), 250 nM of each primer, and 4 µl of KAPA SYBR FAST qPCR Master Mix (Sigma-Aldrich) in a total volume of 8 µl. The PCR program was composed of an initial denaturation at 95 °C for 60 s followed by 40 cycles of denaturation at 95 °C for 10 s, and primer annealing/extension at 60 °C for 30 s. A melting-curve analyses was conducted before concluding the program, which confirmed the purity of all products. Normalised expression levels were calculated according to the geNorm manual (ver. march13, 2007) (Vandesompele et al., 2002). Details are shown in Supplementary qPCR formulas.

Analysis of QAs

Frozen plant tissue was ground in liquid nitrogen using a mortar and pestle. A sample of the powdered material (~100 mg) was weighed, re-suspended in extraction solvent (60% methanol, 0.06% formic acid, and 6–300 µg ml⁻¹ caffeine as internal standard), and vortexed. After sonication in an ultrasonic bath for 15 min, the samples were centrifuged at 20 000 g for 15 min. The supernatants were diluted 1:10 (low-QA samples) or 1:150 (high-QA samples) to give a final solvent composition of 30% methanol, 0.06% formic acid, and 10 µM caffeine. The diluted samples were filtered through a 0.22-µm PVDF filter and stored at -20 °C until analysis.

LC-MS/MS analysis was performed on a Dionex UltiMate 3000 Quaternary Rapid Separation UHPLC+ focused system (ThermoFisher Scientific). Separation was achieved on a Kinetex® 1.7-µm C18 column (100 × 2.1 mm, 1.7 µm, 100 Å; Phenomenex, Torrance, CA, USA), including a SecurityGuard™ C18 guard column (2.1 mm, 2 µm; Phenomenex). Elution proceeded by means of a gradient with 0.3 ml min⁻¹ flow rate, using water as solvent A and acetonitrile with 0.05% formic acid as solvent B. The elution profile was: 0–1 min, 2% B; 1–16 min, 2–25% B; 16–24 min, 25–65% B; 24–26 min, 65–100% B; 26–27 min, 100% B; 27–27.5 min, 100–2% B; 27.5–33 min, 2% B. The temperatures of the LC column and autosampler were maintained at 40 °C and 4 °C, respectively. The injection volume of all samples was 8 µl. The UHPLC was coupled to a Compact microTOF-Q mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ion source (ESI) operated in positive mode. The ion spray voltage was maintained at 4500 V. The dry temperature was set to 250 °C, and the dry gas (nitrogen) flow was set to 8 l min⁻¹. The nebulising gas was set to 2.5 bar and collision energy to 10 eV. Na-formate clusters were used for calibration. MS/MS spectra were acquired in a m/z range from 50–1000 amu at a sampling rate of 6 Hz.

The identification of lupanine was made by comparison to a standard of (+)-lupanine (tartrate salt, purchased from Innosil, Poznan, Poland). For all other QAs, the identification relied on exact masses, fragmentation patterns, UV spectra, and relative retention times.

Metabolite imaging via MALDI-MSI

Cryo-sectioning of plant tissues was performed as previously described (Li *et al.*, 2013). In brief, seed-containing pods were flash-frozen in liquid nitrogen and mounted on the specimen stage of a cryo-microtome (CM3050 S, Leica Microsystems A/S) using water as an adhesive and no further embedding of the tissue. Tissue sections were cut to 30 µm thickness at -20 °C and thaw-mounted on microscope slides, which were subsequently stored at -80 °C until analysis.

At the time of analysis, the samples were taken directly from the -80 °C freezer to a vacuum desiccator in order to avoid condensation of water while reaching room temperature. The samples were sprayed with MALDI matrix as previously described (Wenande *et al.*, 2016), with 300 µl of 2,5-dihydroxybenzoic acid (DHB) dissolved in methanol/water (70:30) being deposited over a circular area of diameter ~3 cm.

After deposition of the matrix, the samples were imaged on a QExactive Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a AP-SMALDI110 ion source (TransMIT, Giessen, Germany). Imaging was performed in positive ion mode using a scan range of m/z 150–600. Using a DHB peak for internal mass calibration, a mass accuracy of ~1 ppm was obtained. The raw data was converted to imzML files (Schramm *et al.*, 2012). MSiReader was used for visualisation and images were created at the exact masses of the targeted analytes using a bin width of 0.002 Da (Robichaud *et al.*, 2013).

Results

We developed an LC-MS-based method for QA analysis. Using this method, we monitored the accumulation of QAs in seeds and pods of a bitter cultivar of NLL (cv. Oskar) throughout development. At each time-point, we also sampled a young leaf close to the inflorescence in order to compare QA profiles. In total, we identified three core QAs (lupanine, 13-hydroxylupanine, and angustifoline) and five esterified QAs [13-*trans*-cinnamoyl-, 13-*cis*-cinnamoyl-, 13-coumaroyl-, 13-(7'-hydroxydihydrocoumaroyl)-, and 13-tigloyl-/angeloyloxylupanine] (Fig. 1A). Details on the assignment of chemical structures are presented in Supplementary Table S2. For all time-points, the seed QA profile was closer to the profile of the pods than it was to the profile of the leaves (Fig. 1B). In general, the three core QAs dominated the profile of seeds and pods, whereas the esterified QAs were dominant for the leaves (Fig. 1B).

With regards to accumulation in seeds and pods, the three core QAs displayed very similar patterns (Fig. 2). In pods, they peaked at 30 DAA and returned to a low, background level at 55 DAA. In seeds, however, the core QAs continued accumulating up to 55 DAA, after which they decreased to around 50% of their maximum levels. In contrast, the patterns of accumulation of the esterified QAs were quite much more varied (Fig. 2). One exception was 13-*cis*-coumaroyloxylupanine, where the accumulation pattern in pods somewhat resembled that of the core QAs. In seeds, however, 13-*cis*-coumaroyloxylupanine only accumulated to low levels and became undetectable from 40 DAA onward. The availability of a standard permitted the quantification of the core QA lupanine in this time series (Fig. 3A, B). At the time of maximum accumulation in seeds (55 DAA), a single pod contained up to 0.2 mg of lupanine in its seeds. At this time-point, the amount of lupanine in the pod tissue itself had decreased from ~0.08 mg at 30 DAA to ~0.002 mg.

We visualised QAs in cross-sections of pods (including seeds) at 30 DAA and 70 DAA using MALDI-MSI (Fig. 4). For comparison, we visualised sucrose by selecting a sucrose-related ion ($m/z=381.079$). Sucrose was present across pods and seeds at both time-points, and its local concentration did not change dramatically between 30 DAA and 70 DAA. In contrast, images obtained by selecting a lupanine-related ion ($m/z=249.196$, $[M+H]^+$) indicated that the local lupanine concentration decreased substantially in pods and increased considerably in seeds between these time-points. The intensity of this ion was particularly high in pods at 30 DAA, where the signal was distributed throughout the different parts of the tissue, including the endocarp. At this time-point, the sucrose-related signal was less strong in the endocarp than in the rest of the pod. The other two core QAs presented a similar localisation to lupanine and displayed very similar changes in signal intensity between 30 DAA and 70 DAA for both pod and seed tissues (Supplementary Fig. S2). In contrast, the esterified QA 13-coumaroyloxylupanine could only be detected in pod tissues at both time-points.

We complemented our QA analysis in seeds and pods with qRT-PCR analysis of the transcript levels of *LDC* (Fig. 3C). In general, *LDC* expression levels were much higher in pods than in seeds, as exemplified by ~100-fold higher expression at 10 DAA. Interestingly, expression peaked in pods at 20 DAA and then decreased rapidly in a linear fashion, reaching background levels similar to the seeds at 55 DAA. The very low expression levels in seeds were maintained throughout their development, with marginally increased levels at 40 DAA and 55 DAA.

In order to assess the level of contribution of *in situ* biosynthesis to seed QA levels, we performed crosses between a bitter variety as father and a sweet NLL variety as mother, and analysed the QA content of the resulting mature F_1 seeds using LC-MS (Fig. 5). As a control, we crossed individuals of the sweet variety with each other using the same crossing protocol. We also included seeds derived from selfed sweet plants and seeds derived from selfed bitter plants. One-way ANOVA indicated that there were no significant differences between F_1 seeds of the bitter male \times sweet female cross, F_1 seeds of the control cross (sweet \times sweet), and seeds derived from selfed sweet plants (Fig. 5, Supplementary Tables S3, S4). This demonstrated a lack of paternal influence on seed QA content, which suggests a low or non-existent contribution of *in situ* QA biosynthesis in the seeds (see Discussion). The experiment was replicated the following year in a different location, producing similar results.

Discussion

We set out to investigate whether *in situ* biosynthesis plays a role in the accumulation of QAs in developing seeds of bitter narrow-leafed lupin (NLL). To do this, we first developed a convenient method for routine QA analysis via LC-MS. Since the 1980s, QA analysis has been traditionally carried out using GC-MS. However, this requires a cumbersome sample preparation protocol that includes aqueous extraction of the plant

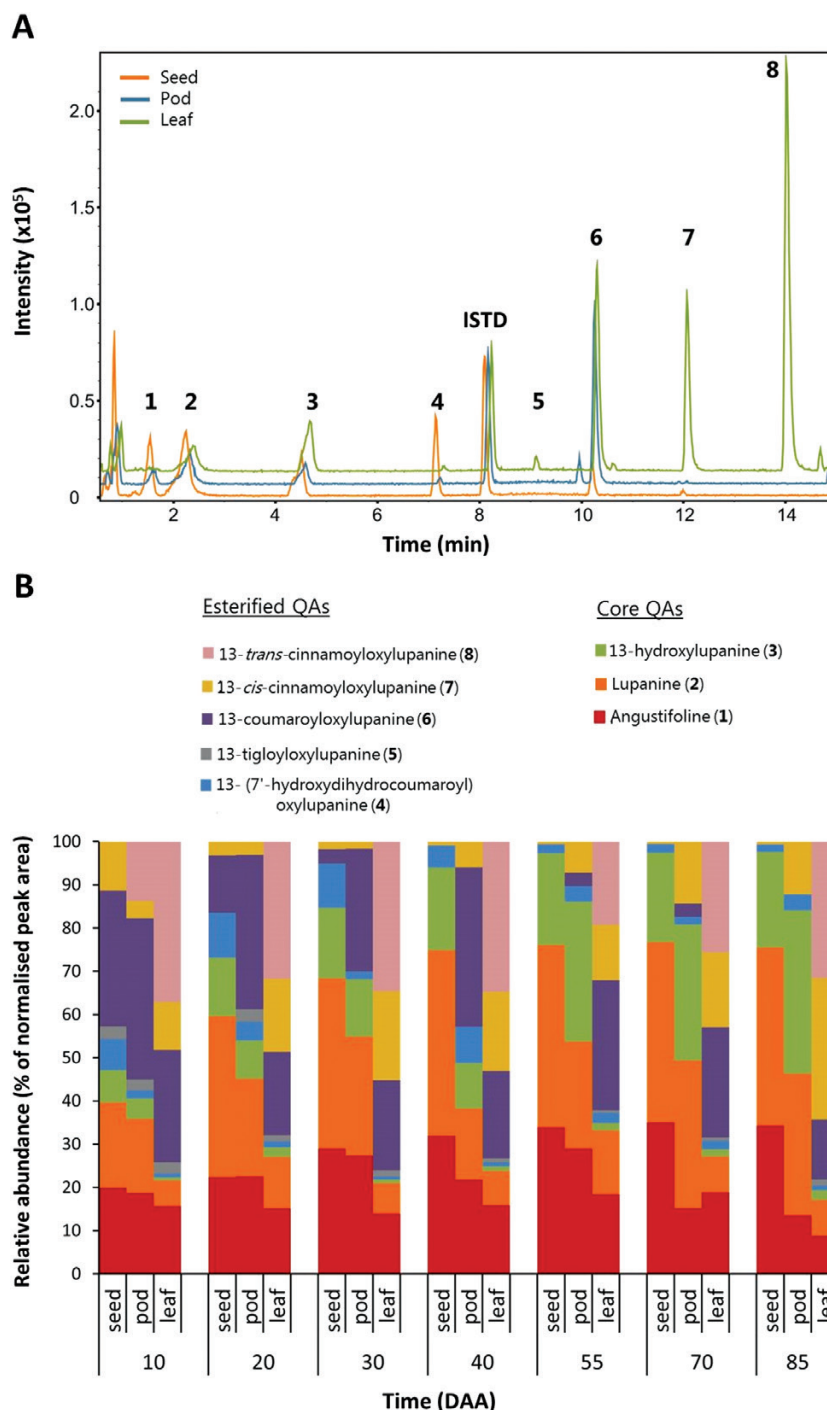


Fig. 1. Profiles of quinolizidine alkaloids (QAs) in seeds, pods, and leaf tissues of bitter narrow-leaved lupin throughout seed development as determined by LC-MS analysis. (A) Total ion chromatograms of representative samples at 30 d after anthesis (DAA). Peak numbers are identified in the key in (B). The peak at 8 min corresponds to the internal standard (ISTD, caffeine). (B) Relative abundance of individual QAs throughout seed development. The data represent the means of 4–6 biological replicates.

tissue under strongly acidic conditions, alkanalisation with a strong base, and a final extraction with a non-miscible organic solvent (Wink and Hartmann, 1982; Frick *et al.*, 2018). The LC-MS analysis that we developed represents a convenient alternative that does not require acid–base extraction during sample preparation, as the original plant extracts can be injected directly into the LC system.

Using this LC-MS-based analysis, we monitored the accumulation of QAs in seeds and pods of bitter NLL throughout

development. We found that the core QAs lupanine, 13-hydroxylupanine, and angustifoline followed similar accumulation patterns, which argues strongly for a similar physiological function. In seeds, the core QAs accumulated continuously until around 55 DAA, which roughly corresponded to the onset of the drying phase (Supplementary Fig. S1). In contrast, pods accumulated core QAs only until around 30 DAA, after which their content decreased to the background level (Fig. 3B). In the pods, the expression pattern of the

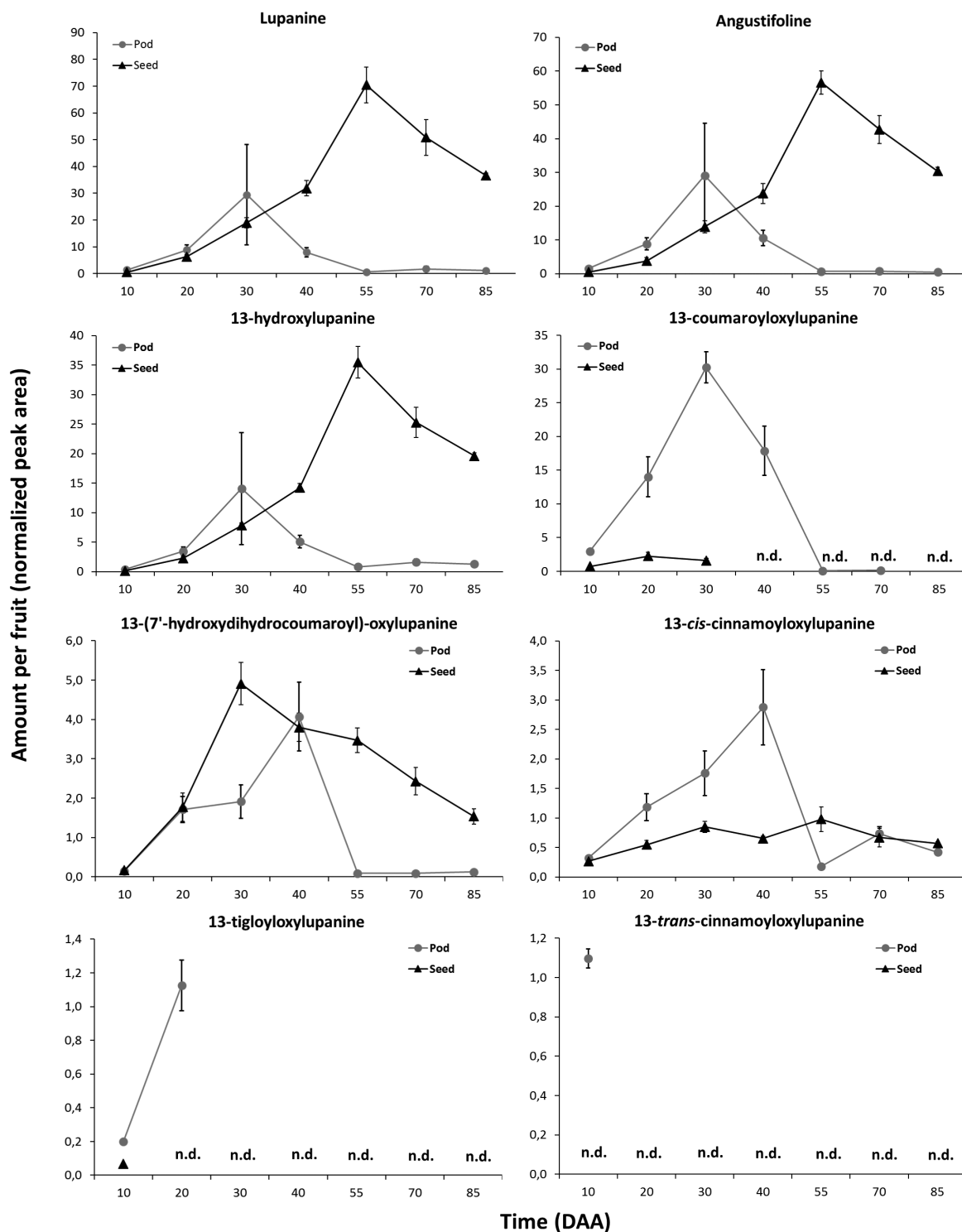


Fig. 2. Patterns of accumulation of individual quinolizidine alkaloids (QAs) in seeds and pods of bitter narrow-leaved lupin throughout development as analysed by LC-MS. Peak areas have been normalised to reflect the total content in seed and pod tissues per individual fruit. Data are means (\pm SE) of 4–6 biological replicates. DAA, days after anthesis.

biosynthetic gene *LDC* resembled the accumulation pattern of the core QAs, with a slightly earlier peak at 20 DAA (Fig. 3C). Interestingly, *LDC* expression levels in the seeds were very low throughout development. Similar low expression was also observed recently by Frick *et al.* (2018) and is suggestive of limited *in situ* biosynthesis and a large contribution of transport to QA accumulation in seeds. However, the extent to which *in situ* biosynthesis occurred in seeds could not be assessed using this data.

In order to gain further insights, we analysed QA levels of F_1 seeds from crosses between a bitter variety as father and a sweet variety as mother (Fig. 5). All commercial sweet cultivars of NLL are homozygous for a particular recessive allele of a locus called *iucundus* (Frick *et al.*, 2017). The identity of the *iucundus* gene has not been determined, but it is known that commercial sweet cultivars have impaired expression of *LDC* in leaves and stems (Bunsupa *et al.*, 2012; Frick *et al.*, 2018).

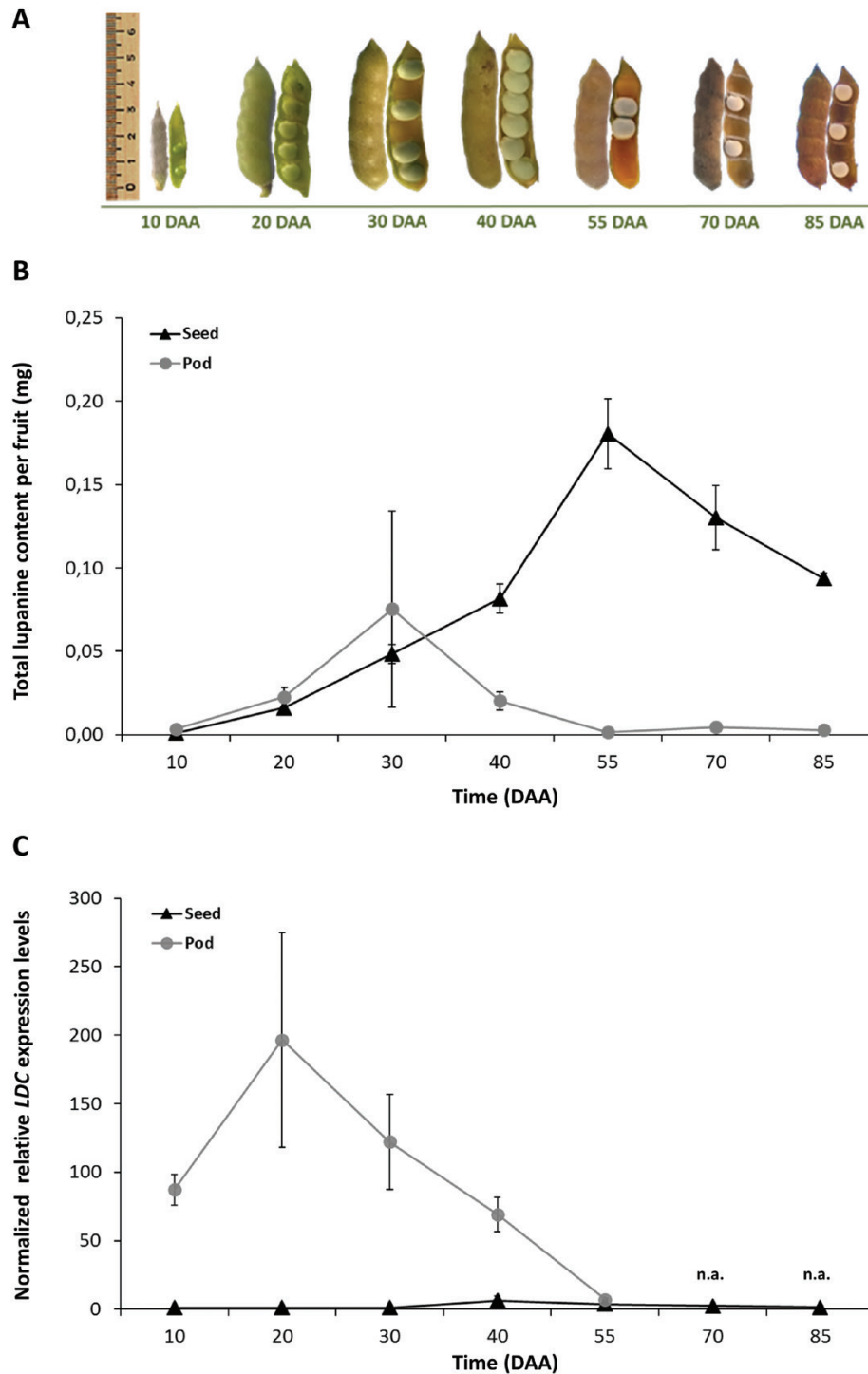


Fig. 3. Lupanine content and expression of *lysine decarboxylase* (*LDC*) in pods and seeds of bitter narrow-leaved lupin throughout development. (A) Representative images of pods and seeds throughout development. The scale is in cm. (B) Total lupanine content for pod and seed tissues per individual fruit as analysed by LC-MS. (C) *LDC* expression throughout development as analysed by qRT-PCR. The normalised relative expression levels have been adjusted so that the mean at 10 d after anthesis (DAA) in seed tissues equals 1; n.a., not analysed. Data are means (\pm SE) for 4–6 biological replicates.

If *iucundus* controls seed QA levels partly through *in situ* biosynthesis, then it would be expected that the F_1 seeds of the crosses described above (grown on a sweet plant and carrying a wild-type copy of *iucundus*) would present increased QA levels compared to a control cross between two sweet plants. Our results indicated that F_1 seeds from a sweet–bitter cross did not differ significantly in QA levels when compared to a control cross (Fig. 5), thus demonstrating that the difference

in seed QA levels between our bitter and sweet varieties was not due to *in situ* biosynthesis. Since the difference in seed QA levels between the parental lines was ~ 70 -fold, we conclude that the contribution of *in situ* biosynthesis in our bitter NLL variety accounted for less than 1.5% of the seed QA levels. However, it should be noted that this crossing experiment did not address the provenance of QAs in seeds of our sweet NLL variety.

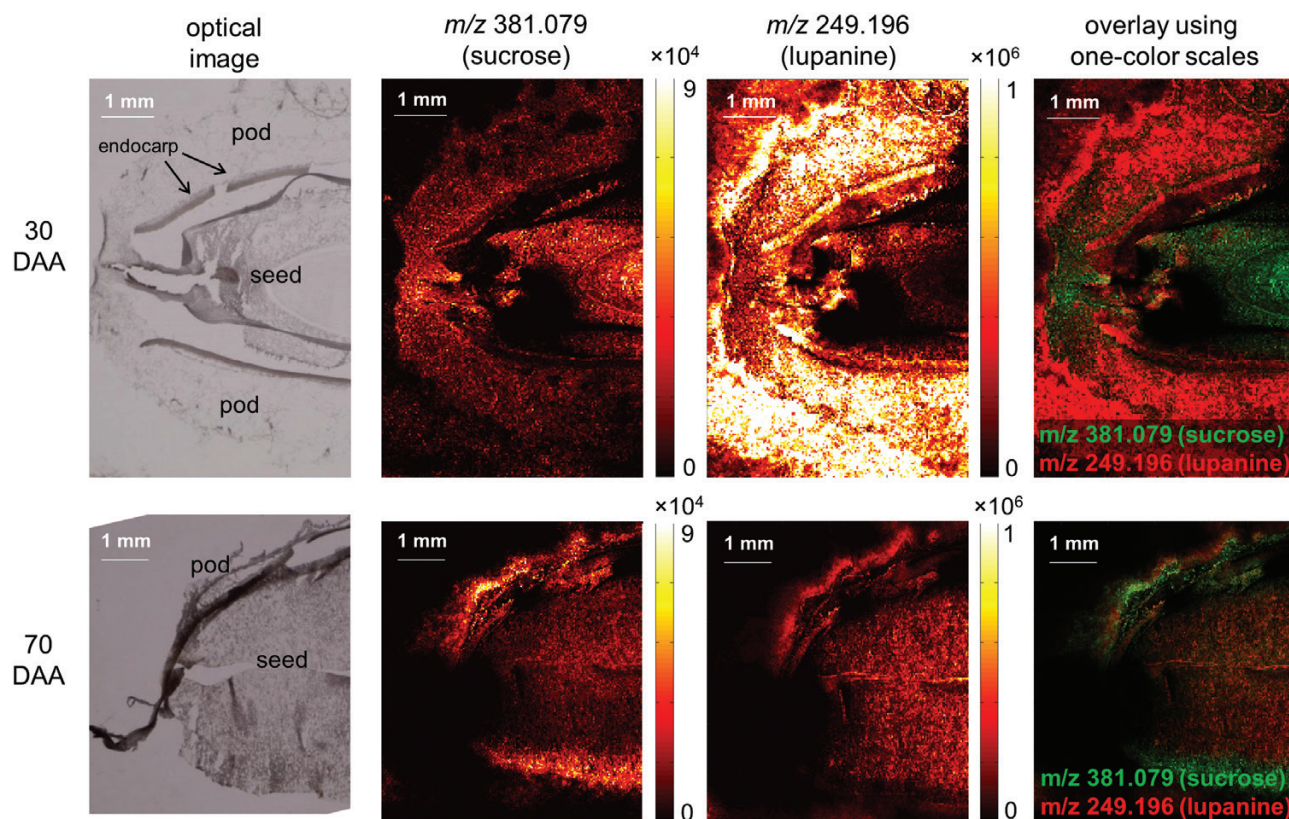


Fig. 4. Distribution of sucrose and lupanine in cross-sections of pods of bitter narrow-leaved lupin as imaged by MALDI-MSI at 30 d after anthesis (DAA) and 70 DAA. At 70 DAA, pods and seeds had already reached maximum dryness (Supplementary Fig. S1C), which explains the thinness of the pod tissue as compared to 30 DAA. The m/z value of 381.079 corresponds to sucrose ($[M+K]^+$) and the value of 249.196 corresponds to lupanine ($[M+H]^+$). The images obtained by selecting these two ions are shown using the multi-color scales presented to the right of each image. The final column shows overlays of images obtained using single-color scales with different colors for sucrose (green) and for lupanine (red). The pixel size is 30 μm .

The influence of the *iucundus* locus on seed QA levels in NLL was first quantified by Harrison and Williams (1983). Even though they did not explicitly state that seed QA levels were maternally derived in NLL, the results of their crossing experiments demonstrated a marked influence of the maternal genotype. It should also be noted that the available literature addressing the issue of transport of QAs into lupin seeds does not take maternal determination into consideration at all (Lee *et al.*, 2007; Frick *et al.*, 2017, 2018). For other plant specialised metabolites, such considerations have been fundamental in establishing early hypotheses about transport into seeds (e.g. for glucosinolates and vicine) (Magrath and Mithen, 1993; Ray *et al.*, 2015). The results of our crossing experiment were in line with those of Harrison and Williams (1983) and offer an increased degree of certainty due to the use of a crossing control, an internal standard, and chemo-typing. Taken together with our results on the accumulation patterns of QAs in seeds and pods and the very low expression of *LDC* in seeds throughout development, we conclude that the vast majority, if not all, of the QA accumulation in seeds of bitter NLL is due to transport processes.

Our experiments do not address the question of which particular tissues provide the seeds with QAs, although it is worth noting that the QA profiles of developing seeds resembled those of the pods much more than those of the leaves. The fact that the pods become almost fully depleted of QAs by the start of the seed drying phase (which corresponded to the peak of accumulation

in seeds) makes it tempting to identify the pods as a significant contributor to seed QAs. Grafting experiments performed by Lee *et al.* (2007) showed that large increases in shoot and phloem QA levels that were observed after grafting a sweet shoot onto a bitter rootstock were only accompanied by modest increases in seed QA levels. These results are consistent with a possible role of the pods in providing the seeds with QAs. Further experiments are needed to determine the relative levels of contribution of different biosynthetic tissues to seed QA levels.

The conclusion that seed QAs are mostly or exclusively transported rather than being produced *in situ* is highly relevant for the design of strategies to obtain a bitter-sweet NLL variety (i.e. bitter in vegetative tissues and sweet in the grain) as it opens the possibility of using transport engineering, in which transport processes are artificially blocked to prevent movement into sink tissues (e.g. seeds). To date, the only long-distance transporters that have been shown to be involved in the translocation of plant specialised metabolites are the glucosinolate transporters *GTR1* and *GTR2*. Simultaneous knock-out of *GTR1* and *GTR2* causes a zero-glucosinolate phenotype in seeds and an over-accumulation of glucosinolates in leaves (Nour-Eldin *et al.*, 2012). Similar results have been obtained by knocking-out multiple GTR homologs in the crop species *Brassica rapa* and *B. juncea* in an elegant example of translational biology (Nour-Eldin *et al.*, 2017). Clearly, however, in order to attempt transport engineering of QAs in NLL, the QA transporters must be first identified and characterised.

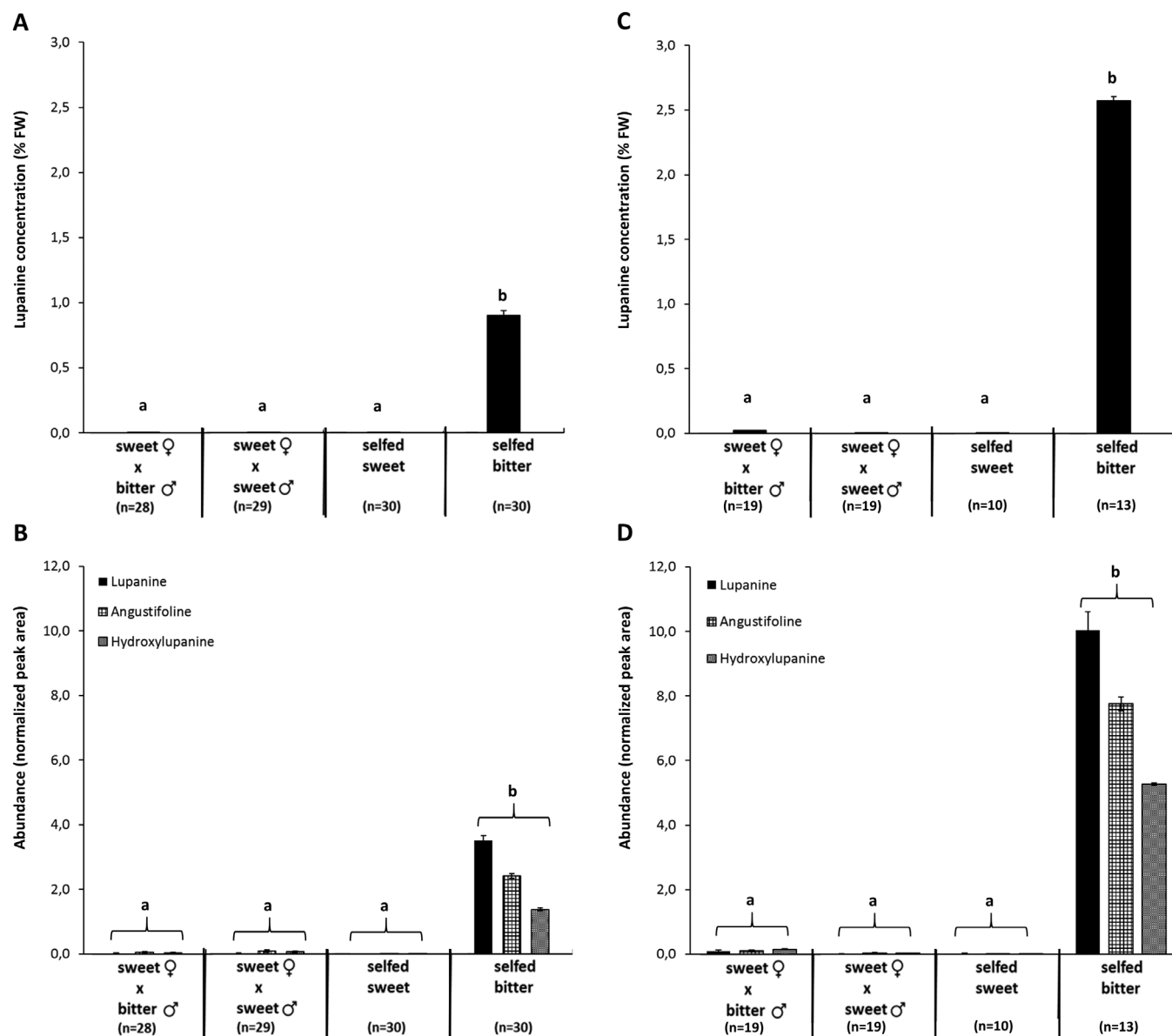


Fig. 5. Lack of influence of the paternal bitter genotype of narrow-leaved lupin (cv. Oskar) on the quinolizidine alkaloid (QA) content of mature F₁ seeds grown on a maternal sweet genotype (cv. Iris), as measured by LC-MS. For comparison, the QA contents of mature seeds derived from selfed sweet and selfed bitter plants are also shown. The data are means (\pm SE) from two independent crossing experiments. (A) Lupanine concentration and (B) abundance of three main QAs from a crossing experiment conducted at Taastrup, Copenhagen in 2016. (C) Lupanine concentration and (D) abundance of three main QAs from a crossing experiment conducted at Frederiksberg, Copenhagen in 2017. n, number of mature seeds analysed per genotype. Different letters indicate significant differences between genotypes as determined using one-way ANOVA ($P < 0.05$).

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. qRT-PCR primers used in this study.

Table S2. Assignment of chemical structures for the analysis of QAs by LC-MS.

Table S3. Statistical analysis of the results of the two crossing experiments corresponding to Fig. 5(A,B).

Table S4. Statistical analysis of the results of the two crossing experiments corresponding to Fig. 5(C, D).

Fig. S1. Fresh and dry weights, and water content of seeds and pods throughout development.

Fig. S2. Distribution of four selected QAs in cross-sections of pods as revealed by MALDI-MSI.

Fig. S3. Content of 13-*trans*-cinnamoyloxylupanine in young leaves of individual F₁ seedlings derived from crosses of female sweet \times male bitter varieties.

qPCR formulas. Calculation of normalised expression levels according to the geNorm manual.

Acknowledgements

We would like to acknowledge Bevan Buirchell (Wise Owl Consulting, Perth, Australia) and Søren Thorndal Jørgensen (Danish Agriculture & Food Council, Copenhagen, Denmark) for valuable advice on crossing NLL varieties. We would also like to thank Karen Michiko Frick (University of Copenhagen) for valuable comments on the manuscript and Davide Mancinotti (University of Copenhagen) for helpful discussions about the chemical structures of the identified QAs. This project was financed by the VILLUM Foundation through a Young Investigator Grant awarded to FGF (Project 15476) and by the Novo Nordisk Foundation through a Postdoctoral Fellowship granted to TY (NNF16OC0019608). The MALDI-MSI instrumentation was funded by the Carlsberg Foundation and The Danish Council for Independent Research | Medical Sciences (grant no. DFF – 4002-00391).

References

- Atnaf M, Yao N, Martina K, Dagne K, Wegary D, Tesfaye K. 2017. Molecular genetic diversity and population structure of Ethiopian white lupin landraces: implications for breeding and conservation. *PLoS ONE* **12**, e0188696.
- Berger JD, Buirchell BJ, Luckett DJ, Nelson MN. 2012. Domestication bottlenecks limit genetic diversity and constrain adaptation in narrow-leaved lupin (*Lupinus angustifolius* L.). *Theoretical and Applied Genetics* **124**, 637–652.
- Berlandier Françoise A. 1996. Alkaloid level in narrow-leaved lupin, *Lupinus angustifolius*, influences green peach aphid reproductive performance. *Entomologia Experimentalis et Applicata* **79**, 19–24.
- Bouchoucha R, Ben Fradj M, Bouchoucha M, Akrouit M, Feki M, Kaabachi N, Raïes A, Slimane H. 2016. Anti-hyperglycemic and anti-hyperlipidemic effects of *Lupinus albus* in type 2 diabetic patients: a randomized double-blind, placebo-controlled clinical trial. *International Journal of Pharmacology* **12**, 830–837.
- Bunsupa S, Katayama K, Ikeura E, Oikawa A, Toyooka K, Saito K, Yamazaki M. 2012. Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in Leguminosae. *The Plant Cell* **24**, 1202–1216.
- Cowling WA, Gladstones JS. 2000. Lupin breeding in Australia. In: Knight R. ed. *Linking research and marketing opportunities for pulses in the 21st century. Current plant science and biotechnology in agriculture*, vol 34. Dordrecht: Springer, 541–547.
- Cowling WA, Tarr A. 2004. Effect of genotype and environment on seed quality in sweet narrow-leaved lupin (*Lupinus angustifolius* L.). *Australian Journal of Agricultural Research* **55**, 745–751.
- de Visser CLM, Schreuder R, Stoddard F. 2014. The EU's dependency on soya bean import for the animal feed industry and potential for EU produced alternatives. *Oilseeds and Fats, Crops and Lipids* **21**, D407.
- Dove ER, Mori TA, Chew GT, Barden AE, Woodman RJ, Puddey IB, Sipsas S, Hodgson JM. 2011. Lupin and soya reduce glycaemia acutely in type 2 diabetes. *The British Journal of Nutrition* **106**, 1045–1051.
- Dracup M, Kirby EJM. 1996. Pod and seed growth and development of narrow-leaved lupin in a water limited Mediterranean-type environment. *Field Crops Research* **48**, 209–222.
- Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrence of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrtosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *Journal of Chemical Ecology* **11**, 1045–1051.
- Erbas M. 2010. The effects of different debittering methods on the production of lupin bean snack from bitter *Lupinus albus* L. seeds. *Journal of Food Quality* **33**, 742–757.
- FAOSTAT. 2018. Food and agriculture organization of the united nations - crop statistics on lupin production. <http://www.fao.org/faostat/en/#data/QC>. (last accessed July 2018).
- Fornasini M, Castro J, Villacrés E, Narváez L, Villamar MP, Baldeón ME. 2012. Hypoglycemic effect of *Lupinus mutabilis* in healthy volunteers and subjects with dysglycemia. *Nutricion Hospitalaria* **27**, 425–433.
- Frick KM, Foley RC, Kamphuis LG, Siddique KHM, Garg G, Singh KB. 2018. Characterization of the genetic factors affecting quinolizidine alkaloid biosynthesis and its response to abiotic stress in narrow-leaved lupin (*Lupinus angustifolius* L.). *Plant, Cell & Environment* **41**, 2155–2168.
- Frick KM, Kamphuis LG, Siddique KH, Singh KB, Foley RC. 2017. Quinolizidine alkaloid biosynthesis in lupins and prospects for grain quality improvement. *Frontiers in Plant Science* **8**, 87.
- Harrison JEM, Williams W. 1983. The control of alkaloid by mutant alleles in *Lupinus albus* and *Lupinus angustifolius*. *Zeitung für Pflanzenzüchtung* **90**, 32–41.
- Kozłowski J, Jaskulska M, Kozłowska M. 2017. The role of alkaloids in the feeding behaviour of slugs (Gastropoda: Stylommatophora) as pests of narrow-leaved lupin plants. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* **67**, 263–269.
- Lee MJ, Pate JS, Harris DJ, Atkins CA. 2007. Synthesis, transport and accumulation of quinolizidine alkaloids in *Lupinus albus* L. and *L. angustifolius* L. *Journal of Experimental Botany* **58**, 935–946.
- Li B, Knudsen C, Hansen NK, *et al.* 2013. Visualizing metabolite distribution and enzymatic conversion in plant tissues by desorption electrospray ionization mass spectrometry imaging. *The Plant Journal* **74**, 1059–1071.
- Lourenço AM, Máximo P, Ferreira LM, Pereira MMA. 2002. Indolizidine and quinolizidine alkaloids structure and bioactivity. In: Atta Ur-Rahman ed. *Studies in natural products chemistry*, vol. 27, part H. Elsevier, 233–298.
- Lucas MM, Stoddard FL, Annicchiarico P, Frías J, Martínez-Villaluenga C, Sussmann D, Duranti M, Seger A, Zander PM, Pueyo JJ. 2015. The future of lupin as a protein crop in Europe. *Frontiers in Plant Science* **6**, 705.
- Magrath R, Mithen R. 1993. Maternal effects on the expression of individual aliphatic glucosinolates in seeds and seedlings of *Brassica napus*. *Plant Breeding* **111**, 249–252.
- Nour-Eldin HH, Andersen TG, Burow M, Madsen SR, Jørgensen ME, Olsen CE, Dreyer I, Hedrich R, Geiger D, Halkier BA. 2012. NRT/PTT transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature* **488**, 531–534.
- Nour-Eldin HH, Madsen SR, Engelen S, *et al.* 2017. Reduction of antinutritional glucosinolates in *Brassica* oilseeds by mutation of genes encoding transporters. *Nature Biotechnology* **35**, 377–382.
- Petterson DS. 2016. Lupin: overview. In: Wrigley C, Corke H, Seetharaman K, Faubion J. eds. *Encyclopedia of food grains*, 2nd ed. Oxford: Academic Press, 280–286.
- Ray H, Bock C, Georges F. 2015. Faba bean: transcriptome analysis from etiolated seedling and developing seed coat of key cultivars for synthesis of proanthocyanidins, phytate, raffinose family oligosaccharides, vicine, and convicine. *The Plant Genome* **8**, 0028.
- Robichaud G, Garrard KP, Barry JA, Muddiman DC. 2013. MSiReader: an open-source interface to view and analyze high resolving power MS imaging files on Matlab platform. *Journal of the American Society for Mass Spectrometry* **24**, 718–721.
- Schopen K, Ewald AC, Johannes BW, Bloch W, Rittweger J, Frings-Meuthen P. 2017. Short-term effects of Lupin vs. whey supplementation on glucose and insulin responses to a standardized meal in a randomized cross-over trial. *Frontiers in Physiology* **8**, 198.
- Schramm T, Hester Z, Klinkert I, *et al.* 2012. imzML—a common data format for the flexible exchange and processing of mass spectrometry imaging data. *Journal of Proteomics* **75**, 5106–5110.
- Sweetingham M, Kingwell R. 2008. Lupins – reflections and future possibilities. In: Palta JA, Berger JB. eds. *Lupins for health and wealth. Proceedings of the 12th International Lupin Conference*, 14–18 Sept. 2008, Fremantle, Western Australia. Canterbury, New Zealand: International Lupin Association, 514–524.
- Taylor CM, Jost R, Erskine W, Nelson MN. 2016. Identifying stable reference genes for qRT-PCR normalisation in gene expression studies of narrow-leaved lupin (*Lupinus angustifolius* L.). *PLoS ONE* **11**, e0148300.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, RESEARCH0034.
- von Sengbusch R. 1942. Süßlupinen und Öllupinen. Teil III. Die Entstehungsgeschichte einiger neuer Kulturpflanzen. *Landwirtschaftliche Jahrbücher* **91**, 793–880.
- Wenande E, Olesen UH, Nielsen MM, Janfelt C, Hansen SH, Anderson R, Haedersdal M. 2016. Fractional laser-assisted topical delivery leads to enhanced, accelerated and deeper cutaneous 5-fluorouracil uptake. *Expert Opinion on Drug Delivery* **14**, 307–317.
- Wink M. 1991. Plant breeding: low or high alkaloid content? In: von Baer D, ed. *Proceedings of the 6th International Lupin Conference*, November 25–30, 1990. Temuco-Pucon, Chile. Temuco, Chile: Asociación Chilena del Lupino, 326–334.
- Wink M, Hartmann T. 1982. Diurnal fluctuations of quinolizidine alkaloid accumulation in legumes and cell suspension cultures. *Zeitung für Naturforschung C* **37**, 369–375.
- Wink M, Hartmann T, Witte L, Rheinheimer J. 1982. Interrelationship between quinolizidine alkaloid producing legumes and infesting insects: exploitation of the alkaloid-containing phloem sap of *Cytisus scoparius* by the broom aphid *Aphis cytisorum*. *Zeitschrift für Naturforschung C* **37**, 1081–1086.
- Yang T, Nagy I, Mancinotti D, Otterbach SL, Andersen TB, Motawia MS, Asp T, Geu-Flores F. 2017. Transcript profiling of a bitter variety of narrow-leaved lupin to discover alkaloid biosynthetic genes. *Journal of Experimental Botany* **68**, 5527–5537.